INCREASED ID1 EXPRESSION IN ALCOHOLIC HEPATITIS AFFLICTED LIVERS

by
Francisco Lopez

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Abstract

Alcohol is a psychoactive substance that can lead to significant organ damage if consumed chronically for long periods of time. The liver parenchyma is the main site of alcohol metabolism, and liver damage can be characterized through a spectrum of pathological conditions termed alcoholic liver disease (ALD). Alcoholic hepatitis (AH) is a late stage of ALD in which an immune response is mounted against the liver parenchyma, leading to poor prognosis and high mortality rates. Crosstalk between nonparenchymal liver cells has been shown to mediate both inflammation and wound healing responses: the liver's resident macrophages, Kupffer cell's, induce transdifferentiation in hepatic stellate cells towards a fibrogenic effector cell lineage. Transdifferentiation has been linked to increases in inhibitor of differentiation 1 (Id1) within hepatic stellate cells. Increases in Id1 are correlated with various models of liver injury, but whether this increase is observed in alcoholic liver injury has yet to be shown. We observed increased expression of Id1 in liver tissue samples from patients experiencing alcoholic hepatitis. Moreover, through western blot analysis of human donor, AH, and alcoholic cirrhosis liver tissue samples we showed the presence of a protein band in AH samples seen only sparsely in the other tissue samples. This protein band is closer to the predicted weight of Id1 which may suggest that the protein is undergoing an activating reaction: dephosphorylation or deubiquitination. We hypothesize that this increase in Id1 is linked to increased cellular oxidative stress brought about by the inflammatory response during alcoholic hepatitis. This information not only confirms increases in Id1 during alcoholic hepatic injury, but it also may shed light on a novel mechanism by which the liver protect itself against oxidative stress.

Primary Reader: Dr. Zhaoli Sun
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Preface and Acknowledgements

This research was done in the Johns Hopkins School of Medicine, Transplant Biology Research Lab in Baltimore Maryland. The majority of the work done for this thesis began after I finished undergraduate. The catalyst for the scrutinization of Id1 in liver tissue samples from patients with alcoholic hepatitis began with a routine immunohistochemical staining screening in which my lab tests different antibodies in a variety of different tissue samples. This was originally where we found the increase of Id1 in alcoholic hepatitis samples. I would like to thank Dr. Brandon Peiffer for patiently instructing me on how to conduct experiments and for pushing me to uphold a standard of excellence in my work. Furthermore, I would like to thank Dr. Zhaoli Sun for providing his insightful expertise throughout the various stages of my project. I extend my gratitude to the rest of the Sun Lab for their consistent support of my work, and their dedication to the field of transplant biology.

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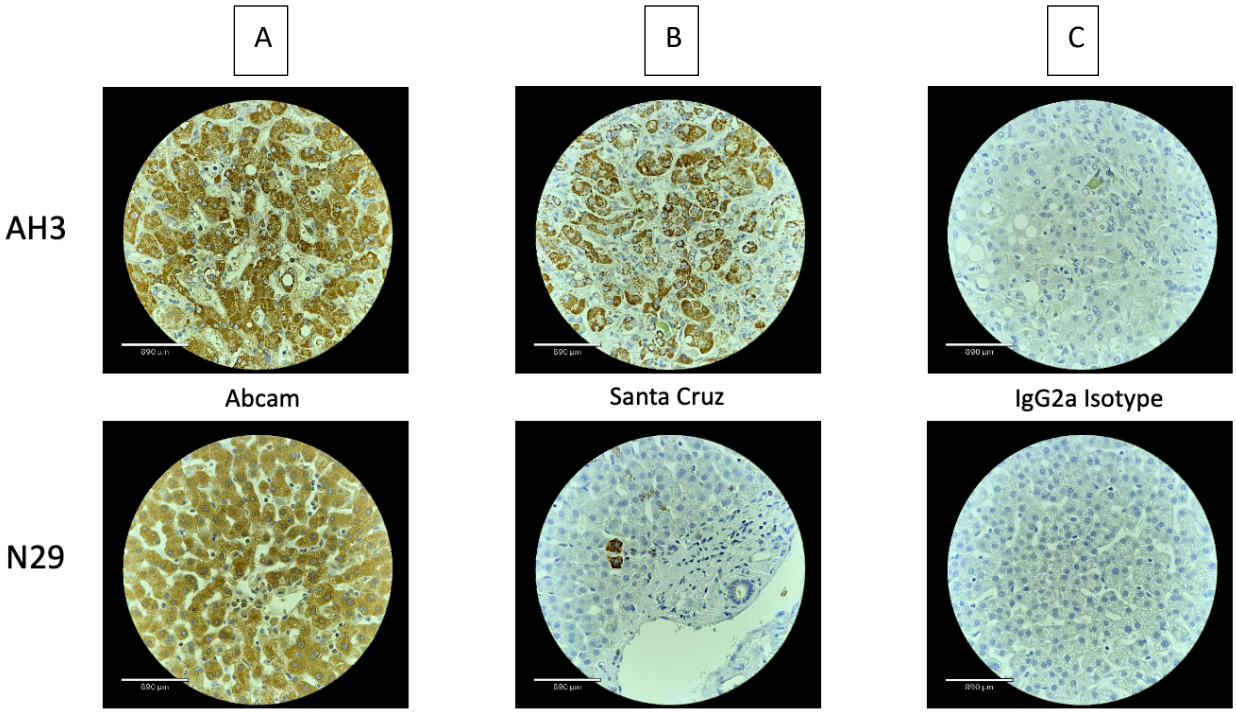
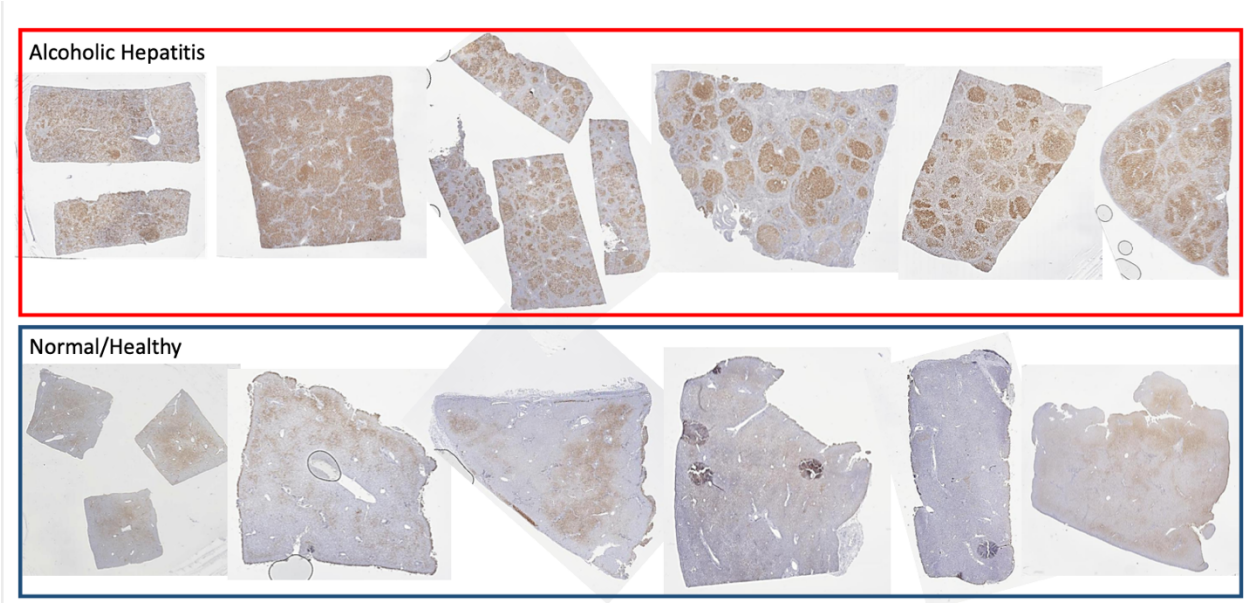


Fig. 1. Testing different Id1 antibodies to gauge differential expression in alcoholic hepatitis (AH, top row) and normal (N, bottom row) liver tissue. Abcam Id1 antibody stained both AH and N tissue to a similar degree which suggests non-specific binding of the antibody since literature consistently states low expression in normal tissue (A). Santa Cruz Id1 antibody-stained AH tissue to a higher degree than normal tissue which suggests more specific binding (B). IgG2a isotype of Id1 antibody used on both tissues as a positive control to demonstrate that binding of antibodies is specific (C).



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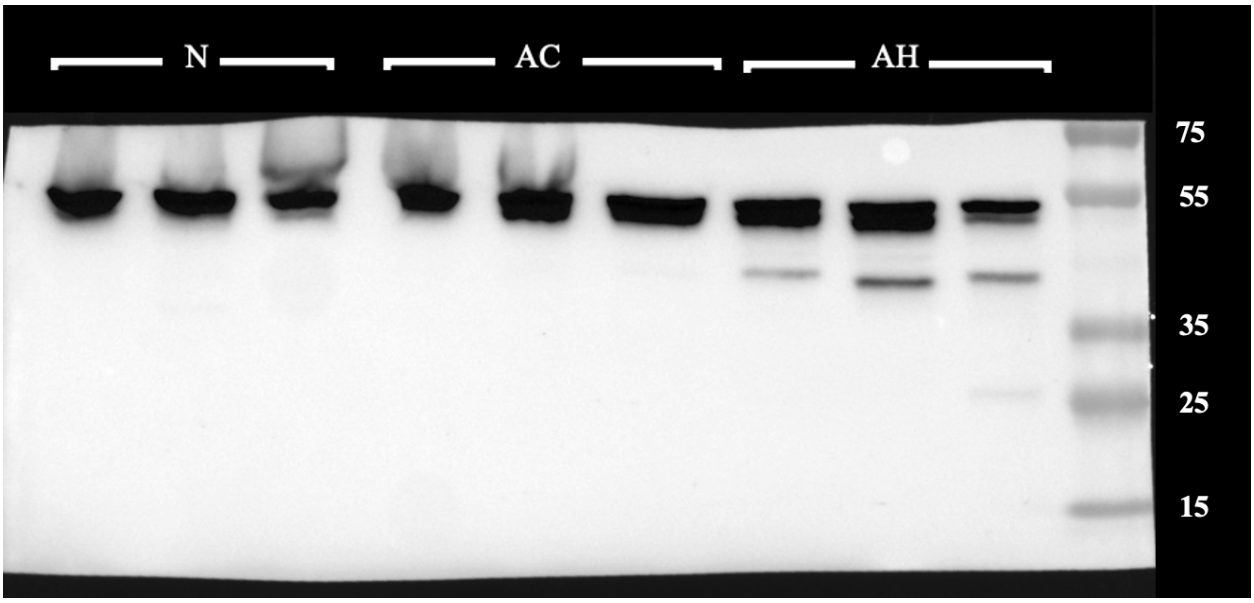

Fig. 3. Western blot analysis of multiple human alcoholic hepatitis (AH), alcoholic cirrhosis (AC), and normal (N) liver tissue samples. Protein extracts were analyzed for the presence of Id1 and there was one extra visible band in AH patients that was not seen in the other experimental groups. Numbers on the right of the molecular ladder correspond to molecular weight in kDa. This suggests that there is some modification occurring to the Id1 protein during alcoholic hepatitis.

Chapter 1: A Review of Alcoholic Liver Disease and Introduction to Id1

Alcohol's Impact

The excessive consumption of alcohol is a healthcare concern that accounted for 3 million deaths worldwide in 2018.[1] The effects of drinking to this degree are imprinted throughout a person's body due to alcohol's damage-inducing properties on all of the body's organ systems. Due to alcohol's effect on the consumer's psychological state, drinking may also indirectly cause a slew of dangerous behaviors. In a 40-year systematic meta-analysis of literature pertaining to alcohol's impact on gun violence it was found that in firearm homicides in the United States reported, 37% of the people that died had drank some amount of alcohol while 30% had drank a heavy amount of alcohol. Similarly, in firearm suicides 35% of decedents drank some amount of alcohol while 25% drank a heavy amount of alcohol.[2] Alongside the staggering mortality, chronic alcohol consumption has been implicated in draining the economy. A study from the CDC estimated that in 2010 the economic cost was close to $250 billion in the United States.[3] It was noted that these numbers would have increased still if the United States had not been in recovery from the recession from 2007-2009.

Alcohol Metabolism

Ethanol is metabolized in the main parenchymal cells of the liver (hepatocytes) which account for 70 percent of the liver's mass.[4] The hepatocyte's chief alcohol metabolizing enzymes are alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1). ADH is the more catalytically efficient enzyme, using NAD^+ as a cofactor generating NADH and acetaldehyde as byproducts. Acetaldehyde is highly toxic and reactive but is very quickly metabolized into acetate by aldehyde dehydrogenase 2 (ALDH2) inside the mitochondria. This reaction also

generates NADH which, when taken together with ADH, can lead to an imbalance in the intrahepatic ratio of NAD^+/NADH (the cellular redox potential). Drastic changes in the cellular redox potential can switch the oxidative metabolism in the liver towards reductive synthesis which causes the formation of fatty acids in the liver.[5] Although CYP2E1 has a catalytic efficiency much lower that ADH, it has a 10-fold higher capacity for binding ethanol. Ethanol directly interacts with the CYP2E1 protein which causes a conformational change that resists degradation, making this enzyme inducible.[6] While this may be beneficial for the metabolism of alcohol, it leads to metabolic tolerance in heavy drinkers–they need to consume more alcohol to have the same intoxication level they had previously.

Alcoholic Liver Disease

Chronic alcohol consumption leads to a spectrum of conditions collectively known as Alcoholic Liver Disease (ALD). The diagnosis for ALD is determined by an established history of heavy alcohol consumption along with physical and laboratory examinations proving that the individual has associated liver disease.[7] Due to the prerequisite of habitual drinking and patient's reluctance to admit such behaviors, ALD is often times not diagnosed. Regardless, each stage has a distinct physiological presentation that can be observed through microscopic analysis of liver tissue sections. In steatosis, liver cells accumulate lipid droplets which can be seen histologically as empty bubbles in their cytoplasm. Alcoholic hepatitis or steatohepatitis present through tissue damage usually seen as ruptures in the tissue or through invasion of immune cells into the liver with a predominance of neutrophils. Fibrosis and its terminal stage cirrhosis are characterized by many thin long cellular projections known as fibroblasts that surround circular patches of functional hepatocytes. The main recommendation for treatment in these diseases is

abstinence. Due to the addictive nature of alcohol, though, this treatment is hard to implement outside of a healthcare setting. For alcoholic hepatitis, corticosteroids or other drugs that aim to curtail the immune system have been implemented; however, these drugs have failed to prove clinically significant increases in survival.[8] Transplantation is another option for the treatment of end stage liver disease.

Steatosis

The most common and earliest stage observed among problem drinkers is steatosis or fatty liver disease; this stage develops in more than 90 percent of problem drinkers and can be developed following binge drinking.[9] Steatosis is characterized by the deposition of fat, seen histologically as lipid droplets in hepatocytes. Initially these lipid droplets are seen in the hepatocytes surrounding the liver's central vein, but as alcohol consumption continues, these lipid droplets can be observed in mid-lobular hepatocytes and those surrounding the hepatic portal vein.[10] As mentioned previously, changes in the cellular redox potential in the liver leads to reductive synthesis which enhances lipogenesis. However, reductive synthesis does not account for the accumulation of fat within the liver. A growing body of research points to multiple mechanisms in the development of fatty liver disease.[5,9]

In line with reductive synthesis, there are other mechanisms that occur in the liver following alcohol consumption which lead to enhanced lipogenesis. One such mechanism is the expression of lipogenic enzymes. Researchers have noted that the transcription factor family, sterol regulatory element-binding proteins (SREBPs), contributes to the accumulation of fat in the liver. These transcription factors regulate lipid synthesis and cholesterol by inducing the expression of more than 30 genes in the liver.[11] In abstinent populations, these transcription

factors are fairly dormant in the liver; however, when one consumes alcohol heavily, the inactive form of these transcription factors, which are normally bound to the nuclear envelope or ER, can undergo processing which allows for the protein to traffic to the nucleus. Once this protein is in the nucleus, it can lead to the transcription of lipogenic enzymes, enhance fatty acid synthesis, and accelerate the accumulation of fat in the liver. Another transcription factor that has been implicated in the acceleration of triglyceride accumulation in the liver is Early growth response-1 (Egr-1). Egr-1 has been established to bind the promoter region of stress-induced genes. One important gene influenced by Egr-1 is tumor necrosis factor-α (TNF- α).[12] In the context of steatosis, TNF-α is considered a lipogenic cytokine due to inducing the maturation of SREBP-1.

Once the lipid droplets have been established in the liver, hepatocytes need to degrade them in order to reverse steatosis. In order to break down lipid droplets, liver cells undergo a process known as lipophagy: fatty droplets are engulfed in a double membrane bound vacuoles termed the autophagosomes, the autophagosomes are trafficked to lysosomes where they fuse, lipases breakdown the triglycerides in the lysosomes, and the free fatty acids can be oxidized in the mitochondria. Chronic alcohol consumption is known to perturb this process at a few different points. Heavy alcohol consumption can lead to faulty biogenesis of lysosomes, resulting in fewer and defective lysosomes.[13] Moreover, the generation of NADH during ethanol oxidation inhibits fatty acid breakdown in the mitochondria. Ethanol oxidation also depolarizes the mitochondria, impairing it's ATP-generating function and leads to a leaky outer membrane.[14] This contributes to inefficient fatty acid breakdown by worsening fatty acid transport to the mitochondria.

Alcoholic Hepatitis

The effects of fatty liver disease can be reversed by simply abstaining from drinking alcohol. However, if consumption continues the disease will progress to alcoholic hepatitis. Alcoholic hepatitis is an inflammatory-type disease with a 28-day mortality ranging from 30% - 50%.[15] The diagnosis for this disease includes both a physical examination and verification through laboratory data. During physical examinations, common clinical manifestations include malnourishment, low blood pressure, tachycardia, jaundice, and with an enlarged, tender liver. Laboratory tests seek to determine levels of bilirubin, sodium, albumin, and white blood cells which have a neutrophilic predominance.[15]

The pathophysiology in the liver is characterized by the swelling and death of hepatocytes, neutrophil infiltration, and the development of aggregates of insoluble proteins known as Mallory-Denk bodies.[16] While neutrophil infiltration is a common motif in alcoholic hepatitis, resident and serum macrophages are thought of as central to the development of the disease. Kupffer cells are the resident macrophage in the liver, they account for 15% of the liver's biomass and 50% of the body's macrophages.[17] Macrophage polarization is important to the induction of an immune response in the liver; they have an M1 state which has been established as proinflammatory and an M2 state which is anti-inflammatory. The functional state macrophages occupy is determined by their microenvironment which is influenced by circulating growth factors, cytokines, pathogen associated molecular pattern, and damage associated molecular patterns.

Nutrient-rich blood travels in a direct route from the intestine to the liver via portal circulation, and due to the extensive microbe profile of the gastrointestinal (GI) tract, countless

pathogens make their way to the liver. Consequently, Kupffer cells have developed tolerogenic properties that prevent from mounting an immune response in the face of circulating pathogens. For this reason, induction of an immune response in the liver requires a second challenge to synergize with excessive alcohol consumption.[18] One such challenge arises from the GI tract. Animal studies confirmed that chronic alcohol consumption leads to overgrowth of bacteria via direct and indirect mechanisms in the GI tract.[19] Furthermore, this dysbiosis is accompanied by increased permeability in the gut epithelium. Maintenance of the epithelium in the gut is immunologically important as it functions as a barrier between us and the environment. It was shown in patients with alcohol dependence that their gut was permeable enough to allow large macromolecules through the intestinal barrier.[20] One important macromolecule, in terms of alcoholic hepatitis, is endotoxin produced by gram-negative bacteria. This molecule is known to cause severe disease such as sepsis once in the circulation. In the liver, however, alcohol sensitized Kupffer cells can use their pattern recognition receptors to detect endotoxin. Engagement of these receptors, CD14 and TLR4, with their ligand results in the transcription of proinflammatory cytokines and subsequent activation of the immune cell. Once activated, Kupffer cells release these proinflammatory cytokines such as TNF-α, interleukins, chemokines, as well as produce reactive oxygen species that cause oxidative stress, and subsequently, the release of more cytokines.[21]

The release of these factors into the liver has deadly consequences. Hepatocytes are normally immune to TNF-α, but after being sensitized by alcohol, these cells undergo apoptosis. Interestingly, the phagocytosis of apoptotic bodies by macrophages functions as an alternative mechanism that converts macrophages into their proinflammatory functional state.[15] Chemokines released by Kupffer cells can be sensed by other immune cells in circulation such as

lymphocytes and neutrophils, attracting them to the site of inflammation. Their effector functions in the liver synergize with the reactive oxygen species production from Kupffer cells and alcohol metabolism in general which exacerbates oxidative stress.

Fibrosis

Liver fibrosis is the result of chronic liver injury followed by abnormal deposition of extracellular matrix proteins. These proteins interfere with the architecture of the liver through forming fibrous scars, and the development of hepatocyte nodules is what characterizes the terminal stage of fibrosis–cirrhosis. Changes to the cellular architecture of the liver results in increased resistance to blood flow, hepatic insufficiency, and hypertension in the portal venous system. Due to the insidious nature of this disease, diagnosis and appropriate scaling of fibrosis is of paramount importance. In this regard, biopsies of liver tissue remain the golden standard; however, there are limitations to this approach. Biopsies are an invasive procedure that leads to pain and major complications in 40% and 0.5% of cases, respectively.[7] Correctly characterizing the disease relies upon the features of the sample obtained, and therefore, there is a risk associated with sampling error or interobserver variation. While analyzing the concentrations of specific proteins such as N-terminal peptide of type III collagen or inhibitor of metalloprotease type 1 (TIMP-1) can offer a good indication of advanced cirrhosis or no fibrosis, they often times lack the ability to characterize intermediate levels of the condition reliably.[7]

Hepatic stellate cells exhibit the secretion of extracellular matrix components into the liver. Under normal physiological conditions, hepatic stellate cells are storage sites for vitamin A and lie dormant within the space of Disse, the space in between hepatic sinusoids and the liver parenchyma. During hepatitis, Kupffer cells secrete signals, namely PDGF, which activate

quiescent hepatic stellate cells. Activated hepatic stellate cells transdifferentiate toward myofibroblast-like cells and contribute to the inflammatory response by secreting more chemokines, cytokines, and adhesion factors that attract immune cells from the periphery.[22] Attracted leukocytes target hepatocytes while also activating more stellate cells, leading to a positive feedback loop. At the resolution of inflammation, Kupffer cells release anti-inflammatory cytokines such as prostaglandin D2 which stimulates the wound healing, extra cellular matrix deposition by hepatic stellate cells.[17] The accumulation of extracellular matrix components is mediated by increased secretion and decreased breakdown. Breakdown of the ECM by matrix metalloproteases is stifled by the increased expression of their inhibitors (TIMPs) during fibrosis.[23]

Inhibitor of Differentiation

The helix-loop-helix (HLH) transcription factors are a large family of proteins that rely primarily on their highly conserved HLH domains for protein-protein interactions. Their HLH domain consists of two amphipathic α-helices connected by a loop and gives these proteins the capability to homo- or heterodimerize with other HLH proteins in order to influence gene expression. The resulting fold between dimers is a non-covalent, parallel, left-handed four-helix bundle and in basic (b)HLH dimers this conformation forms a tweezer-like structure which is ideal for DNA binding.[24] Class V HLH proteins are unique in that they lack a DNA-binding motif; consequently, these proteins sequester class I and II bHLH proteins, forming non-DNA binding dimers and act as dominant negative regulators of bHLH-mediated gene expression. There are a few pathways that lead to the expression of Id1, the most notable being the bone morphogenic protein (BMP) and nuclear factor Y complex (NFY-c) signaling pathways.[24] BMPs

are a subgroup of the TGFβ superfamily and multiple have been implicated in the expression of Id1.[25] Once a BMP protein interacts with its corresponding receptor (Type I, Type II, and co-receptor Endoglin), the cytoplasmic tail of the receptor becomes phosphorylated. This phosphorylation event leads to autophosphorylation amongst the receptors which attracts and phosphorylates the SMAD1/5/8 complex. This phosphorylated complex can bind SMAD4, allowing the complex to localize to the nucleus where it can bind, amongst other regions, the promoter of Id1 and positively induce expression. The NFY-c binds to the CCAAT box on the promoter of Id1 which was shown to induce expression of the protein. In the NTera2 human embryonic cell line, NFY-promoter interaction decreased as retinoic acid causes differentiation in the cell.[24] This could be attributed to the loss of NFY-c at the protein level as the cell differentiates.

Id1 and The Cell Cycle

One key set of Id1 binding partners, from which the protein received its name, are Cdk inhibitors. Id proteins antagonize the induction of gene expression in differentiation-associated genes such as p15, p16, and p21.[24] The expression of these Cdk inhibitors is tied to bHLH proteins which can dimerize with Id1, leading to regulation of the cell cycle in the G1 phase. Crosstalk between p53 and Id1 has been reported as well: Id1 impairs the p53-mediated response to DNA damage while p53 upregulates the bHLH transcription factor DEC1 that downregulates Id1.[24] This crosstalk has been implicated in the regulation of cell-cycle arrest and senescence versus cell-cycle progression.[24]

Chapter 2: Increased Expression of Id1 in Alcoholic Hepatitis Liver Samples

2.1 Introduction

The excessive consumption of alcohol is a healthcare concern that accounted for 3 million deaths worldwide in 2018.[1] Among people between the ages of 20 and 39, approximately 13% of deaths were attributable to alcohol consumption.[1] The damaging effects of alcohol on the liver lead to the development and progression of Alcoholic Liver Disease (ALD). Progression through ALD revolves around the histological changes of hepatocytes as a result of constant alcohol exposure. The most common and earliest stage observed among problem drinkers is steatosis; this stage develops in more than 90 percent of problem drinkers and can be developed following binge drinking.[9] Steatosis is characterized by the deposition of fat, seen histologically as lipid droplets in hepatocytes. Initially these lipid droplets are seen in the hepatocytes surrounding the liver's central vein, but as alcohol consumption continues, these lipid droplets can be observed in mid-lobular hepatocytes and those surrounding the hepatic portal vein. Steatosis can be reversed if alcohol consumption ceases; however, increased exposure to alcohol in the liver leads to alcoholic steatohepatitis which is characterized by the swelling and death of hepatocytes, neutrophil infiltration, and the development of aggregates of insoluble proteins known as Mallory-Denk bodies. In this stage liver damage is inflicted by the activation of the immune system, namely Kupffer cells, the resident macrophages in the liver. During steatohepatitis, these cells are found to secrete cytokines that not only kill hepatocytes, but also recruit more immune cells to infiltrate the liver. Fibrosis and its terminal stage cirrhosis refer to scaring of the liver tissue. These stages are characterized by the unusual deposition of extracellular matrix proteins.

There is evidence of crosstalk between different hepatic cell types during the pathogenesis of alcoholic hepatitis and fibrosis.[22] Hepatic stellate cells reside in a quiescent state in the space of Disse, the space between the liver parenchyma and sinusoid, and function as a major storage site for vitamin A. Under inflammatory conditions, these cells are known to transmit proinflammatory cytokines in response to stimulation from the sinusoid resulting in amplification of inflammation. At the resolution of an inflammatory response, Kupffer cells release prostaglandin D2, an anti-inflammatory cytokine, which stimulates a wound healing, fibrogenic response in hepatic stellate cells.[17] This wound healing response is characterized by transdifferentiation of the stellate cell towards a myofibroblast-like cell morphology and abnormal secretion of extracellular matrix proteins. It has been reported that an increase in inhibitor of DNA binding 1 (Id1) is accompanied by the transdifferentiation of hepatic stellate cells.[17]

Id1 is a class V helix-loop-helix (HLH) protein, and unlike basic (b)HLH proteins, Id1 lacks an N-terminal associated DNA-binding domain. HLH proteins function as important regulators of gene expression. (b)HLH proteins rely on non-covalent interactions between their HLH motifs for homo- and heterodimerization, and if the two conjoined proteins contain DNA-binding domains, this quaternary protein structure forms a tweezer-like structure which allows the protein to bind DNA and influence gene expression. Since Id1, along with other class V HLH proteins, do not contain DNA-binding domains, they function as dominant negative inhibitors of HLH-regulated gene expression. An increase in Id1 is known to play an important role in many different cellular processes in liver cells including liver regeneration, dedifferentiation of hepatic cancer cells, and fibrogenesis.[4, 26] Although previous reports have found increases in the expression of Id1 correlated to hepatic injury models such as hepatitis C virus[27], hepatitis B

virus[28], and hepatocellular carcinoma[29], this trend has not been evaluated in alcoholic liver injury models. In this paper, we show that there is an increase in the expression of Id1 correlated with alcoholic hepatitis. This information not only validates that Id1 is a commonly induced gene during hepatic injury, but it also may shed some light into the crosstalk between nonparenchymal liver cells and parenchymal liver cells.

2.2 Methods

Immunohistochemical staining

To determine the expression of Id1 in both normal and alcoholic hepatitis (AH) liver tissues, we used immunohistochemical staining. Human donor and AH liver tissues were collected during liver transplantation and stored in formalin for 3 days. These samples were then processed, embedded in paraffin wax, cut into 4 μm sections, and mounted on positively charged slides. The sections were incubated with a rabbit monoclonal antibody against Id1 in a dilution of 1:250. There is also uncertainty that the monoclonal antibody that is used for these experiments targets only what we intend it to; therefore, we used an isotype of our antibody (IgG2a) to determine specificity. More 4 μm sections of the same samples were cut and incubated with IgG2a for comparative analysis.

SDS-PAGE and Western Blot

Western blots were used to determine the amount of Id-1 present in different liver tissues. Normal donor, AH, and alcoholic cirrhosis (AC) liver tissues were homogenized and centrifuged to isolate proteins. The protein concentration from each protein lysate was standardized using the BioRad DC Protein Assay. Five microliters of each protein lysate were separated in denaturing

conditions using BioRad Mini-PROTEAN® TGX 10% gels. Separated proteins were transferred to a nitrocellulose membrane using polyacrylamide gel electrophoresis (PAGE). The membrane was washed using distilled water before incubating in TBS-T/10% BSA for 30 minutes. Following incubation in the blocking buffer, the mouse anti-Id1 monoclonal antibody was added into the blocking solution in a 1:500 dilution and stored overnight on a rocker in -4 °C. The next day, the primary antibody was removed and washed three times with TBS-T for five min per wash. The membrane was then incubated in anti-mouse secondary antibody in a 1:5000 dilution for one hour. Following three more washes, the membrane was visualized using enhanced chemiluminescence substrate.

Immunoprecipitation

In order to isolate and analyze our protein of interest we will use the Pierce Crosslink Magnetic IP/Co-IP Kit (Thermo Scientific). To begin, we incubated rabbit anti-Id1 monoclonal antibodies with magnetic beads at a dilution of 10 µg per 100 µL for 15 min. Following washes to remove unbound antibodies, we incubated the antibody bound beads with a DSS crosslinker (10X molar excess relative to beads) for 30 min. Following purification, we stored the beads in a 4 °C. Human donor and AH liver samples were homogenized and centrifuged in order to obtain a protein lysate. We used the BioRad DC Protein Assay to standardize protein concentrations among the different samples. We incubated 500 µL of diluted lysate with crosslinked beads for 1 hour at room temperature. The proteins were then isolated by washing the beads with elution buffer and collecting the beads with a magnetic stand, separating the remaining liquid. We then ran more SDS-PAGE and Western Blot to confirm that our protein of interest was present in the bound antigens.

2.3 Results

Increased Id-1 Expression During Liver Damage

Following incubation with a rabbit monoclonal antibody against the protein Id-1, healthy donor tissue (termed normal. Fig. 2.) showed modest staining. This suggests that during normal physiological conditions, the liver does not express much of the Id-1 protein. Incubation with the same monoclonal antibody in alcoholic hepatitis liver samples shows visibly increased tissue staining. This illustrates that during chronic liver damage, the cells of the liver parenchyma (hepatocytes) change their expression profile to translate more of the Id-1 protein.

Altered Id-1 Protein Structure

Western blot analysis of healthy human donor liver tissues showed that the majority of the Id-1 protein was around 50 kDa (Fig. 3.). In other experimental groups, alcoholic hepatitis and alcoholic cirrhosis, a band at this size was present. While there may be differences in tissue staining between donor and alcoholic hepatitis groups, there is some commonality observed in protein size. However, in the alcoholic hepatitis group, there was another band present that was not present to the same extent in other groups. This band was observed at around 25 kDa which may suggest protein dimerization, a common conformation found amongst the bHLH proteins. The western blot was carried out under reducing conditions, though, which eliminates the possibility of non-covalent protein interactions. The predicted weight of the Id-1 protein is around 16 kDa which suggests that there may be some covalent modification that is slowing the migration of this protein, leading to its appearance higher than expected on the membrane.

2.4 Discussion

Further experimentation is required to confirm the protein structure of the different Id1 bands. Due to the benign gel shift, though, we predict that it may be related to a mixture of post-translational modifications to the protein, namely ubiquitination and phosphorylation. Moreover, we hypothesize that the observed increase in Id1 protein levels during alcoholic hepatitis is part of a stress response mechanism aimed at protecting liver cells from the oxidative stress caused by the inflammatory response.

Id1 has been linked to a variety of cellular processes such as proliferation, regeneration, hematopoiesis, cell-cycle regulation and differentiation.[30-32] The role of Id1 in other forms of hepatitis is somewhat controversial. One report described that hepatitis B viral components, such as its core and x proteins, are directly involved in destabilizing Id1 and reducing its transcription in the livers of mice.[33] However, another publication shows that Id1 increases as hepatitis B viral protein x expression increases in immunohistochemical stains of human liver tissue.[28] Increased Id1 expression was also reported in biopsies of liver tissues from patients with chronic hepatitis C viral infection.[27] All of these papers share the conclusion that chronic infection with either virus leads to profibrogenic responses and hepatocellular carcinoma (HCC). Immunohistochemical staining of HCC tissue samples have consistently shown to express high levels of Id1[29]; thus, increased expression of Id1 during infection with hepatitis virus is expected, as it is a common cause of HCC. Expression of Id1 in HCC has been linked to metabolic reprogramming of cancer cells through the regulation of c-Myc, leading to aerobic glycolysis and glutaminolysis.[34] The expression of Id1 has also been reported in other liver cell types, conferring pro-fibrogenic responses in hepatic stellate cells.[10] Wiercinska et. al. elucidated that ALK1/SMAD1 phosphorylation and subsequent Id1 expression was necessary for the

transdifferentiation of hepatic stellate cells to myofibroblasts. Myofibroblasts secrete matrix proteins into the liver, the characteristic sign of fibrosis. Altogether, our findings correspond with ongoing literature that Id1 is positively regulated in livers damaged through hepatitis. Whether the same result would be seen in the latter stages of ALD such as fibrosis, cirrhosis, or HCC requires further experimentation.

Interestingly, western blot analysis of Id1 protein in liver tissues harvested from patients with alcoholic hepatitis, alcoholic cirrhosis, and normal tissue revealed the presence of a band corresponding to a lower molecular weight in the alcoholic hepatitis experimental groups (Fig. 2.). One key feature of the bands was the gel shift, there appeared to be multiple different bands observed. The western blot was conducted under reducing conditions which suggests that this shift may be caused by some covalent modifications. However, the molecular weight of the new band in the alcoholic hepatitis experimental group is closer to the predicted molecular weight of Id1. This suggests that the band corresponds to the removal of covalent modifications or unmodified Id1 protein. It has been reported that Id1, much like other regulators of the cell cycle, has a short half-life, being degraded by the 26S proteasome.[35] Proteins that traffic to the proteasome can be poly-ubiquitinated or phosphorylated because these covalent modifications function as tags for proteasome-dependent degradation. We speculate that the band we observe in the alcoholic hepatitis experimental groups corresponds to unmodified Id1 protein. Thereupon Id1 may be afforded a longer half-life where it can carry out its dominant negative transcriptional suppression activity.

The most predominant form of cellular stress during alcoholic hepatitis (AH) is the increased oxidative burden placed upon hepatocytes. Before the induction of AH, liver cells sustain considerable changes to their redox potential due to the metabolism of alcohol.

Activation of Kupffer cells during AH leads to the production of reactive oxygen species (ROS) which increases oxidative stress. Moreover, release of cytokines by the liver's resident macrophages stimulates the recruitment of other immune cells such as neutrophils and circulating macrophages whose effector functions lead to the production of more ROS. Aside from apoptosis, salvage mechanisms such as MAPK cascades become active in response to increased levels of ROS. The expression of early growth response-1 (Egr-1) has been documented as a downstream target of ERK1/2 pathway in response to oxidative stress.[36] An Egr-1 consensus sequence has been identified in the promoter of Id1[37] and other studies have shown a positive correlation between Egr-1 and Id1 expression.[38] Moreover, the expression of Id1 was shown to be increased in the pancreatic beta cell line MIN6 when cultured with H_2O_2. This increase in Id1 expression was also shown *in vivo* in the pancreatic cells of diabetic mice which was correlated with a global antioxidant response. When the researchers knocked out Id1/3, they observed a decrease in the expression of antioxidant enzymes and an increased level of ROS and apoptosis. These results led them to conclude that Id1 was, in fact, an important factor in the response to oxidative stress.[39]

In conclusion, we hypothesize that increased ROS production during AH leads to the induction of Egr-1 which then stimulates the expression of Id1 in an attempt to protect cells from damage associated with increased oxidative burden. Id1 leads to cellular proliferation and the expression of antioxidant genes which make it an important regulator of oxidative stress. We believe that the increased Id1 protein levels are linked to the appearance of the lighter band in the western blots. Future studies should aim to assess the levels of ROS and antioxidant enzymes in Id1 knockout cell lines and mice in order to validate the proposed mechanism. The proposed Id1 stress response mechanism has been shown to be true in other organ systems but has never been

validated in the liver. This evidence gives insight into the protective mechanisms of the liver which may provide useful information when studying other protective mechanisms such as the wound healing fibrogenic response.

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Curriculum Vitae

Francisco Lopez

505-440-2949 | Flopez6@jhu.edu
3333 N Charles St, Baltimore, MD 21218

EDUCATION

Johns Hopkins University **Baltimore, MD**
Master of Science in Biology *Expected May 2021*

Johns Hopkins University **Baltimore, MD**
Bachelor of Science in Molecular and Cellular Biology, Minor: Spanish for professions *May 2020*
GPA 3.5 | **MCAT**: 514
Relevant Coursework: Advanced Spanish, Conversational Spanish, Translations, Spanish Practicum: Community Based Learning

West Mesa High School **Albuquerque, NM**
High School Diploma
June 2016
GPA 4.4 | **ACT**: 32

STUDENT LEADERSHIP AND VOLUNTEERING EXPERIENCE

Esperanza Center **Baltimore, MD**
Volunteer *January 2020 – March 2020*

- Aided medical professionals with Spanish interpretation to make the clinic environment more efficient and welcoming.
- Scheduled appointments for immigrant patients to meet with medical specialists and ensured that they were notified and received the proper paperwork.
- Helped with signing in patients, preparing their file for their appointment, running through medical notes to ensure they understand how to take medicine and where they can acquire it, and provided coupons to help with the cost.

Lambda Epsilon Mu **Baltimore, MD**
Vice president; Stem-leads chair; Public relations chair *August 2017 – May 2020*

- Responsible for maintaining business relationships, grant writing, and leading various community service engagements.
- Drafted multiple flyers and actively update social media accounts to improve membership and
  notify current members of available opportunities. Since appointed membership doubled.
- Wrote grants to fund medical related opportunities for the organization.
-

Tutorial Project **Baltimore, MD**
Tutor; Organizer *September 2016 – May 2020*

- Matched with and tutored a Baltimore elementary school student to strengthen skills in math, reading, and writing.
- Managed multiple tutor-tutee pairs in order to help streamline learning goals appropriate for student success. This includes testing the student to elucidate academic needs, communication with tutors to inform them of upcoming events in the organization,

determining the progress of the student throughout the program via drafting and testing learning goals, and communication with parents to ensure the student retains knowledge.

STEM-Leads **Baltimore, MD**
STEM-LEADS chair; Vice President *August 2017 – May 2020*

- Presented on various STEM-related topics to a public Baltimore high school with a majority of the students identifying as underrepresented populations to foster an interest in seeking higher education.
- Worked as part of a team to develop presentations and interactive group activities for the students.
- Wrote grants that were not only able to fund the program, but also to supply students with SAT/ACT prep materials and laptops.

Professionals in Health Podcasting Series **Baltimore, MD**
Content creator *January 2019 – Present*

- Worked with a team of students to provide the Johns Hopkins community with informative interviews with medical professionals from various different fields, medical students, and other professionals with medical related occupations.
- Aided in the transfer to a more accessible format, from a student group related website to iTunes and Spotify.
- Wrote grants that fund our monthly streaming service to maintain our platform and host events.

Hop-In **Baltimore, MD**
Program Mentor *May 2017 – May 2018*

- Counseled and mentored a group of matriculated freshmen by providing academic and personal development skills and support, serve as liaison to student support services.
- Implemented various academic, cultural, and service based enrichment events/programs for freshmen mentees throughout academic year with a team of other mentors.

Upward Bound Math and Science **Albuquerque, NM**
Resident Assistant *June 2018 – August 2018*

- Counseled and mentored a group of high school students by providing relevant information for college admissions: taught ACT material, provided free exams, edited college essays, and drafted four-year plans.

PROFESSIONAL EXPERIENCE

Johns Hopkins Transplant Surgery Research **Baltimore, MD**
Graduate Research Assistant *June 2019 – Present*

- Analyzed the effects of chronic alcohol consumption on the liver cell expression profile in human tissue samples.
- Revised standardized lab protocols for Western blot, immunohistochemical staining, and SDS-PAGE experiments.
- Conducted experiments for a new drug and elucidated its effects on stem cell mobilization for wound healing studies in diabetic animal models.

Our Daily Bread: Spanish SNAP Hotline **Baltimore, MD**
Volunteer Coordinator *November 2020 – Present*

- Helped Spanish-speaking immigrants collect evidence in order to complete an application to receive food stamps
- Recruited and trained ten new volunteers, growing the Hotline's workforce by 300%
- Devised practical solutions to track volunteer work and maintained orderly processes

Johns Hopkins Transplant Surgery Department **Baltimore, MD**
Observer *December 2018 – January 2019*

- Observed the daily rotations of various transplant surgeons, which include post-operational evaluation, transplant recipient determination, review of case studies, and surgeries, for a total of 120 hours.
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Johns Hopkins Emergency Department **Baltimore, MD**
Observer *December 2018 – March 2019*

- Observed the daily rotations of an attending physician in the emergency department, which include a diverse set of patient interaction that range from both physical and mental ailments, for a total of 40 hours.

Stop Glaucoma: Wilmer Eye Institute **Baltimore, MD**
Undergraduate Research Assistant *June 2019 – November 2019*

- Preform free standard eye exams to screen for glaucoma in various unique communities in Baltimore, provide free reading glasses, prescription lenses, and follow-up appointments for patients in need of health resources.
- Completed survey with patients to identify the different rates of various eye-related illnesses and access to healthcare among different communities in Baltimore.

AWARDS

Johns Hopkins University: Bloomberg Scholar **Baltimore, MD**
Merit-based Academic Scholarship *May 2016 – May 2020*

Johns Hopkins University: Dean's List **Baltimore, MD**
Maintained 3.5 GPA or higher *January 2017 – May 2020*

Johns Hopkins Alumni Association Grant **Baltimore, MD**
Earned $2,000 *January 2020*

Idea Lab's Grant for Diversity Innovation: STEM-LEADS **Baltimore, MD**
Earned $10,000 *April 2018*

SKILLS

Computer Skills: MS Excel, Word, and PowerPoint
Language Skills: Fluency in English and Spanish